REMARKS

Claims 1, 3-4, 6-7, 19 and 23-27 constitute the claims pending and under consideration.

Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Office Action will be addressed below in the order they appear in the Office Action.

Information Disclosure Statement

Applicants note with appreciation that the Examiner has considered the Information Disclosure Statement filed on June 8, 2009.

Claim Rejections – 35 U.S.C. § 112, First Paragraph

Claims 1, 3, 4, 6, 7, 9, 19, and 23-27 are rejected under 35 U.S.C. § 112, as allegedly failing to comply with the enablement requirement. Applicants respectfully traverse.

Applicants reiterate the arguments already made of record and contend that the claims are enabled throughout their scope. Independent claim 1 is directed to a method for reducing a T-cell mediated immune response in an animal by inhibiting an interaction between a dendritic cell and a T cell, comprising administering to an animal in need of reducing said immune response an antibody which binds to a protein with the amino acid sequence of SEQ ID NO: 2 (DC-SIGN) on the surface of a dendritic cell, wherein said antibody reduces one or more interactions between a dendritic cell and a T cell thereby reducing said immune response in the animal, and wherein the animal is not infected with HIV.

Specifically, the Examiner asserts that "[t]he complexity of the art and of the currently claimed invention is that an antibody that binds SEQ ID# 2 can both increase and decrease the immune response. While applicant argues that the specification includes two different inventions, other than the description in the disclosure or the preamble in the claims, the difference in immune response in the methods [is not] differentiated by distinct method steps." See Office Action, page 3, lines 12-16.

Applicants respectfully disagree. While the specification teaches that certain embodiments of the application relate to methods of increasing an immune response in an animal, the immune response is against a specific antigen by presenting an antigen (or one or more antigenic parts

thereof) to dendritic cells in a form that can bind to the C-type lectin receptors on the surface of dendritic cells, which then process and present the antigen to the T-cells, thereby causing an immune response against the antigen (e.g., page 15, lines 3-26). The specification further teaches that "the antigen can be attached to (e.g. fused with or covalently bonded to) an antibody directed against the C-type lectins, preferably a monoclonal antibody such as AZN-D1 and AZN-D2 mentioned above; or to a part or fragment of such an antibody as described above" (e.g., the paragraph bridging pages 15 and 16). Briefly, an antibody may be attached to a specific antigen and this antibody-antigen conjugate may be used for targeting the antigen to dendritic cells, and subsequently inducing an immune response against the specific antigen.

By contrast, the claimed invention (e.g., claim 1) is directed to a method for reducing a T-cell mediated immune response in an animal by using a naked antibody that binds to the DC-SIGN on the surface of a dendritic cell and blocks the interaction of the dendritic cell with a T cell. Such method results in an inhibition of T-cell mediated immune activity. Contrary to the Examiner's assertion, one of skill in the art could readily differentiate these two inventions, which require different forms of an antibody (the first requires an antibody-antigen conjugate, whereas the second uses a naked antibody) and cause different outcomes (the first causes an increase in a specific immune response, whereas the second causes a decrease in immune activity).

The Examiner further asserts that "Applicants' argument that in vitro examples are enough when there is good correlation is not persuasive. First, the portion of the MPEP quoted refers to small pharmaceutical molecules not antibodies, and the method requires binding to a cell and then triggering further action in the form of a reduced immune response. As previously stated, the examples and the prior art do not show this function." See Office Action, the paragraph bridging pages 3 and 4.

Applicants respectfully disagree. First of all, the quoted section of the MPEP (MPEP § 2164.02) itself is not directed to small molecules. Even if the particular case law being cited (*Cross v. Iizuka*, 753 F.2d 1040 (Fed. Cir. 1985)) involves a small molecule, the MPEP makes no distinction between a small molecule or a large molecule. In addition, Applicants submit that the distinction drawn by the Examiner between a small molecule and a large molecule (e.g., an antibody) is irrelevant to the enablement rejection. It is well known that antibodies bind to their

antigens with exquisite specificity. The specification also demonstrates that the disclosed antibodies bind to the DC-SIGN, and inhibit the interaction between the DC-SIGN on the surface of dendritic cells and an ICAM receptor on the surface of T cells. The Examiner did not explain why the type of molecule, e.g., an antibody as compared to a small molecule, would make any difference in evaluating enablement. Second, Applicants point out that the Examiner has incorrectly characterized the claimed method by stating that "the method requires binding to a cell and then *triggering further action* in the form of a reduced immune response" (page 3, last line, emphasis added). In the claimed method, an anti-DC-SIGN antibody binds to its target (DC-SIGN represented by SEQ ID NO: 2), thereby blocking the DC-SIGN from further interactions with a T-cell. With DC-SIGN blocked, the further actions (e.g., interaction with a T-cell) that are required for an immune response cannot occur, resulting in a decreased immune response. Thus, the binding of the antibody does not trigger further action as the Examiner asserts; to the contrary, it blocks further action. Accordingly, in the claimed method, an antibody may actually be better than a small molecule since the antibody may block an interaction between DC-SIGN and a T-cell more efficiently due to steric interference. All that is required is for the antibody to bind to DC-SIGN thereby blocking interaction of DC-SIGN with T-cells.

Applicants reiterate that *in vivo* efficacy data are not required to enable an *in vivo* use and that *in vitro* test results are generally predictive of *in vivo* testing results. See MPEP § 2164.02; also see *Cross v. Iizuka*, 753 F.2d 1040, 1050 (Fed. Cir. 1985). Scientifically sound explanations, backed by *in vitro* testing, are widely accepted as sufficient evidence to support claims drawn to subject matter commensurate in scope with that support. See *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995). The instant application provides several examples, including *in vitro* data derived from cell based assays, to support the claimed subject matter. For example, Example 2 demonstrates that an antibody against DC-SIGN can inhibit the interaction between DC-SIGN (on the surface of the dendritic cells) and an ICAM receptor (on the surface of the T cells). See e.g., paragraph [0100] and Figures 2A and 2C. Example 6 further demonstrates that: (1) anti-DC-SIGN antibodies prevented the clustering of dendritic cells with ICAM-3-expressing K562 cells (Figure 6B); (2) anti-DC-SIGN antibodies inhibited the clustering of dendritic cells with PBLs (which include T-cells) (Figure 6C); and (3) most importantly, anti-DC-SIGN antibodies inhibited the activation of T-cells

when T-cells were mixed with dendritic cells (Figure 6D), as discussed in Example 6 and Example 7. These assays are representative of what occurs *in vivo*, i.e., there are interactions between DC-SIGN and T cells which initiate an immune response (see e.g., paragraph [0093] of the instant application). The application also teaches and enables reducing an immune response by inhibiting an interaction between DC-SIGN and a T cell. Therefore, the application teaches and enables the method as recited in independent claim 1. A person of ordinary skill in the art can make and use the claimed methods based on the *in vitro* data and other teachings of the specification, without undue experimentation.

In addition, Applicants emphasize that various publications have demonstrated that *in vitro* results are predictive of *in vivo* results (see the various Exhibits as previously submitted). In particular, it is reported that an anti-DC-SIGN antibody, when administered *in vivo* in a nonhuman primate model, successfully bound to DC-SIGN expressed on the surface of dendritic cells *in vivo*. See, for example, Pereira et al., J. Immunother. 30:705-714 (2007) (previously submitted as Exhibit 2). Applicants **resubmit a copy of Pereira et al.** since the Examiner asserts that this publication was not found attached as an exhibit. In view of the examples provided by the specification and the additional evidence as previously submitted by Applicants (e.g., Perira et al.), one of skill in the art would recognize that an anti-DC-SIGN antibody can successfully bind to DC-SIGN *in vivo* and block interaction between DC-SIGN and a T-cell *in vivo*, thereby resulting in a decreased immune response *in vivo*.

Further, Applicants submit that the Examiner has the burden to provide reasons for a conclusion of a lack of correlation with *in vitro* testing or an *in vivo* animal model (see MPEP 2164.02). In this case, the Examiner has merely asserted that the level of unpredictability in the art is high and on this basis concludes that one skilled in the art would not associate *in vitro* efficacy with *in vivo* treatment. The Examiner has not provided any specific reasons to doubt that the *in vitro* data correlate with *in vivo* efficacy. The Examiner has not provided any evidence to suggest that the instantly claimed methods would not be operative with respect to reducing T-cell mediated immune response *in vivo*, thereby failing to meet the burden of establishing a *prima facie* case of lack of enablement.

Finally, Applicants point out that the scope of enablement only needs to bear a "reasonable correlation" to the scope of the claims. *See, e.g., In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970); MPEP 2164.08. There is no requirement in U.S. patent law that the claimed invention must work 100% of the time under all conceivable circumstances. "All that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art." MPEP 2164.08. Applicants submit that the data presented in the specification more than satisfy the "reasonable correlation" of the *in vitro* working examples with the claimed method as required by case law.

In sum, Applicants submit that the pending claims are enabled throughout their scope. Applicants respectfully request that the Examiner reconsider and withdraw the enablement rejection.

Related Applications

Applicants wish to bring the Examiner's attention to co-pending, commonly assigned Application Serial No. 11/977,151, filed October 22, 2007. Applicants invite the Examiner to consider previous, on-going, and future prosecution in the co-pending application. Applicants note that the most recent action in the co-pending application is a Response to non-Final Office Action filed on November 12, 2009.

Applicants also wish to bring the Examiner's attention to U.S. Patent No. 7,541,032 (issued June 2, 2009), U.S. Application Serial No. 12/387,112 (a Non-Final Office Action mailed September 18, 2009), and U.S. Application Serial No. 10/524,394 (Notice of Allowance mailed on October 28, 2009).

CONCLUSION

In view of the above remarks, Applicants believe the pending application is in condition for allowance. Early and favorable consideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Applicants believe no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. **18-1945**, under Order No. **ALXN-P02-089** from which the undersigned is authorized to draw.

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Respectfully submitted,

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In Vivo Targeting of DC-SIGN-positive Antigen-presenting Cells in a Nonhuman Primate Model

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Summary: In vivo targeting of antigen-presenting cells (APCs) with antigens coupled to antibodies directed against APC-specific endocytic receptors is a simple and a promising approach to induce or modulate immune responses against those antigens. In a recent in vitro study, we have shown that targeting of APCs with an antigen coupled to an antibody directed against the endocytic receptor DC-SIGN effectively induces a specific immune response against that antigen. The aim of the present study was to determine the ability of the murine antihuman DC-SIGN antibody AZN-D1 to target APCs in a cynomolgus macaque model after its administration in vivo. Immunohistochemical analysis demonstrated that macaques injected intravenously with AZN-D1 have AZN-D1–targeted APCs in all lymph nodes (LNs) tested and in the liver. DC-SIGN–positive cells were mainly located in the medullary sinuses of the LNs and in the hepatic sinusoids in the liver. No unlabeled DC-SIGN molecules were found in the LN of AZN-D1–injected macaques. Morphologic criteria and staining of sequential LN sections with a panel of antibodies indicated that the DC-SIGN–targeted cells belong to the myeloid lineage of APCs. In conclusion, this is the first study that shows specific targeting of APCs in vivo by using antibodies directed against DC-SIGN.

Key Words: anti-DC-SIGN antibody, cynomolgus macaque, antigen-presenting cells, lymph nodes, liver

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Dendritic cell (DC)-specific intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) is a mannose-specific type II membrane C-type lectin with a short amino-terminal cytoplasmic tail and a single carboxyl-terminal carbohydrate-recognition domain (CRD). DC-SIGN is expressed by macrophages and DCs.[1,2] Both cells types are antigen-presenting cells (APCs), which can capture and process antigens in the peripheral blood and tissues, migrate to the lymphoid tissue and present the antigens to resting T cells. DC-SIGN has been described as a receptor for several pathogens including HIV-1.[3] Importantly, DC-SIGN, and also another C-type lectin receptor DEC-205, can bind and endocytose antigens.[4,6] These antigens enter the endocytic compartments, are loaded onto major histocompatibility complex (MHC) class II molecules and stimulate proliferation of antigen-specific $CD4^+$ T cells.

The potent capacity of APCs to generate immune responses has led to the development of vaccination strategies that involve ex vivo loading of autologous APCs with tumor or pathogen-derived antigens and their subsequent administration to patients.[7,8] Unfortunately, this ex vivo approach is very laborious and expensive. An attractive alternative is to load the APCs with the antigen(s) directly in vivo. In principle, APCs can be targeted with antigens coupled to recombinant viral vectors, microparticles, receptor ligands, or receptor-specific antibodies. Targeting APCs in vivo with specific antibodies is an attractive method because of their specificity, applicability in the clinical setting, and because many antibodies that target a cell surface receptor can induce receptor-mediated endocytosis. Several studies have shown that antibodies directed against the endocytic receptor DEC-205 can target antigens to DCs in an in vivo mouse model[6,9,12] and, when combined with a maturation stimulus, can allow the induction of an immune response against those antigens. In a recent in vitro study, we have shown that targeting of keyhole limpet hemocyanin to human APCs via an antibody directed against the endocytic receptor DC-SIGN effectively induced the proliferation of keyhole limpet hemocyanin-specific $CD4^+$ and $CD8^+$ T cells.[5] Mice have 5 distinct DC-SIGN homologs that exhibit expression profiles distinct from that of human DC-SIGN, hampering the interpretation of in vivo studies in mice.[13] Therefore, the aim of this study was to determine the

specificity of the murine antihuman DC-SIGN monoclonal antibody AZN-D1 to target DC-SIGN-positive APCs in a nonhuman primate model in vivo. AZN-D1 has been shown previously to recognize rhesus macaque and chimpanzee DC-SIGN.[14] Because of the limited availability of rhesus macaques, we used cynomolgus macaques (*Macaca fascicularis*) that are genetically, physiologically, and behaviorally similar to rhesus macaques as a model. Therefore, we first demonstrated that cynomolgus macaques also express DC-SIGN, which is highly homologous to rhesus macaque and human DC-SIGN. We also showed that AZN-D1 could detect the expression of DC-SIGN in cynomolgus macaques. Finally, we evaluated the specificity of AZN-D1 for APCs in cynomolgus macaques in vivo.

MATERIALS AND METHODS

Antibodies

Preparations of AZN-D1 and AZN-D3 (mIgG1, antihuman DC-SIGN)[1] were produced from hybridoma supernatants, or from ascites fluid after a 1:5 dilution with Pierce Binding Buffer (Pierce, Rockford, IL). Briefly, diluted antibody was loaded onto Protein A Sepharose 4 Fast Flow columns (Amersham, Buckinghamshire, UK), the columns washed with binding buffer, the bound antibody eluted with 100 mM of glycine, pH 3.5 and neutralized with Tris buffer. Absorbance was determined at 280 nm and chosen fractions were pooled before dialysis into phosphate-buffered saline (PBS). The overall average yield was 3.9 mg of purified antibody/mL initial ascites fluid. AZN-D1 was biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce) according to the manufacture's protocol. The rabbit anti-CD3 polyclonal sera, mouse monoclonal anti-CD20 (clone L26, mIgG1), and anti-CD68 (clone EBM11, mIgG1) were purchased from DAKO Cytomation, Glostrup, Denmark). Mouse monoclonal anti-DC-LAMP (clone 104.G4) and biotinylated mouse monoclonal anti-HLA-DR/DP (MHC class II; clone Q5/13) were purchased by Immunotech (Marseille, France) and Leinco Technologies (St Louis, MO), respectively. Mouse monoclonal anti-CD31 (clone WM-59) was purchased from Pharmingen (San Jose, CA). Mouse IgG1 and IgG2a (R&D systems, Abingdon, UK) and total rabbit IgG (Jackson ImmunoResearch; Westgroove, PA) were used as controls. Biotinylated horse antimouse and biotinylated goat antirabbit were purchased by Vector Laboratories (Burlingame, CA).

Animal and Human Tissues

Nine cynomolgus macaques (*M. fascicularis*) were used in this study. The experiments described were performed using a minimal number of nonhuman primates, in accordance with ethical policies established by the United Kingdom Animals (Scientific Procedures) Act (1986) (the Act) and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule I to the Act, issued under Section 21 of the Act. Eight cynomolgus macaques were purpose-bred and obtained from Vietnam via a commercial supplier (Belgrave Services). The experiments were performed at Huntingdon Life Sciences, Woolley Road, Alconbury, Huntingdon, Cambridgeshire, England. The in-life experimental procedures were subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act (1986) (the Act); and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule I to the Act, issued under Section 21 of the Act. Eight animals were treated intracutaneously with 2.5% oxazolone on days 3, 4, and 5. Oxazolone is a chemical allergen, which can maximize the migration of local APCs from the injection site to the draining (auricular) lymph node (LN). Four of these macaques also received intravenous injections of 3 mg/kg/d of AZN-D1 on days 1 to 7. The animals were euthanized on day 8 with a lethal dose of pentobarbitone anesthesia and exsanguinated by excision of the carotid blood vessels. Tissues were collected immediately after euthanasia and specified organs were fresh-frozen. Frozen samples were placed on corks, snap frozen in isopentane, cooled in liquid nitrogen, and stored frozen (approximately −70°C). For comparison, frozen inguinal LN and liver tissues from an untreated (naive) macaque were obtained from the German Primate Center (Göttingen, Germany) euthanized owing to poor prognosis after trauma. This untreated control animal was of similar health and age and was housed under similar conditions as the 8 animals described above. Animal care and handling was performed under the German Animal Protection Law and in accordance with guidelines of the German Primate Center (Göttingen, Germany). As the expression pattern of APCs in the LNs of untreated macaques was similar to those observed in the LNs of macaques that treated with oxazolone, both untreated and oxazolone-treated macaques will be referred to as control cynomolgus macaques.

A sample of human liver was obtained from 1 patient, a 78-year-old man undergoing a partial hepatic resection as treatment for colon carcinoma, following national guidelines regarding the use of human tissues. All tissues were sectioned at 5 μm for immunohistochemistry and eosin and hematoxylin staining.

Cloning and Sequencing of Cynomolgus Macaque DC-SIGN

Frozen liver and LNs tissues from control cynomolgus macaques were homogenized and lysed in 1 mL TRIzol (Invitrogen Life Technologies, Gaithersburg, MD) according to the manufacturer's guidelines. Total RNA was dissolved in diethylpyrocarbonate-treated water and 12 μg of RNA were used for the synthesis of complementary DNA (cDNA). Afterwards, a polymerase chain reaction (PCR) was performed with the following primers: 5′-GTGCTGAGGAGCAGAACTTC-3′ (sense) and 5′-GCAGATCCAGAATTTGGCAAG-3′ (antisense). These primers were complementary to regions

conserved between the human and rhesus DC-SIGN genes. cDNA was subjected to 35 cycles to detect DC-SIGN mRNA. Amplified DNA was extracted and purified from a standard agarose gel in TAE (0.04 M of Tris, 0.001 M of EDTA-Na2-salt, and 0.02 M of acetic acid) buffer with a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) according to the manufacturer's guidelines. The PCR product was then inserted into a pGEM-T Easy Vector (Promega, Madison, WI) according to the manufacturer's guidelines and DH5α competent bacteria were transformed with the construct by heat-shock. Positive clones were selected by PCR using the same primers as described above. Plasmid DNA was purified from clones that contained the insert using a QIAprep Miniprep (QIAGEN) according to the manufacturer's guidelines. Sequence analysis was performed on 400 ng of plasmid DNA using 50 ng of T7 promotor or M13 reverse primers.

Immunohistochemistry

Cryosections of tissues were fixed at room temperature (RT) with either cold 100% acetone for 10 minutes or 4% paraformaldehyde for 30 minutes, depending on the primary antibody used. After washing with PBS, tissue sections were incubated for 1 hour at RT with 10% normal serum from the species in which the second antibody was raised. The serum was then discarded and incubated overnight at 4°C with the primary antibody. For the liver tissue samples, endogenous biotin was blocked by 15-minute incubation with avidin, followed by 15 minutes incubation with biotin (Avidin/Biotin Blocking kit, Vector Laboratories, Burlingame, CA). Liver tissue sections were washed with PBS before and after the biotin block. Afterwards the tissue sections were washed with PBS and incubated with the secondary antibody for 30 minutes at RT. After extensive washing with PBS, the final staining was performed with the ABC-PO/ABC-AP Vectastain kit (Vector Laboratories, Burlingame, CA). AEC (Zymed, Invitrogen Immunodetection, San Francisco, CA) and diaminobenzidine (ImmunoLogic, Duiven, The Netherlands) were used as substrates for peroxidase and Fast Red (Sigma-Aldrich, Zwijndrecht, The Netherlands) was used as substrate for alkaline phosphatase. Tissue sections were counterstained with hematoxylin and washed extensively with water and either mounted with Kayser's glycerol (Merck, Darmstadt, Germany) or, when diaminobenzidine substrate was used, were dehydrated and mounted with Entellan (J.T. Baker, Chemicals BW, Deventer, The Netherlands).

RESULTS

Cynomolgus Macaque DC-SIGN CRD is Highly Homologous to Rhesus Macaque and Human DC-SIGN

As cynomolgus macaque DC-SIGN had not been previously sequenced, and as the epitope recognized by AZN-D1 contains a valine residue at position 351 (V351) of the human DC-SIGN CRD, we amplified the cynomolgus macaque DC-SIGN CRD from LN and liver tissues with primers complementary to regions conserved between the human and rhesus DC-SIGN genes. Sequence analysis of the protein sequence from cynomolgus macaque DC-SIGN CRD indicated that it is 100%, 98%, 97%, and 79% homologous to pig-tailed macaque, rhesus macaque, human DC-SIGN, and human L-SIGN, respectively (Table 1). In addition, the CRD region containing V351 is conserved in cynomolgus macaque but not in rhesus macaque DC-SIGN, suggesting that AZN-D1 can bind to cynomolgus macaque DC-SIGN.

High Expression of DC-SIGN in Lymphoid Tissues of Cynomolgus Macaques

Although the expression of DC-SIGN in rhesus macaques and chimpanzees has been extensively described,[14–16] the expression of DC-SIGN in cynomolgus macaques has not. Therefore, we first analyzed the expression of DC-SIGN on frozen LN sections from control cynomolgus macaques. In addition, antibodies against CD20 (Fig. 1A) and CD3 (Fig. 1B) were used to stain B cells and T cells, respectively. Isotype-matched controls did not demonstrate significant background staining (data not shown). Similar to findings in rhesus macaques,[15] large numbers of DC-SIGN–positive cells were present in the medullary sinuses and some DC-SIGN–positive cells were present in the afferent sinuses and in the paracortical area of several LNs from control cynomolgus macaques (Figs. 1D–H).

TABLE 1. Comparison of the Protein Sequence of Human, Rhesus Macaque, Pig-tailed Macaque, and Cynomolgus Macaque DC-SIGN CRD

Cynomolgus macaque	AEEQNFLQLQSSRSNRFTWMGLSDLNHEGTWQWVDGSPLLPSFKQYWNKGEPNN[V]GEEDC	60
Pig-tailed macaque	AEEQNFLQLQSSRSNRFTWMGLSDLNHEGTWQWVDGSPLLPSFKQYWNKGEPNNVGEEDC	60
Rhesus macaque	AEEQNFLQLQSSRSNRFTWMGLSDLNHEGTWQWVDGSPLLPSFKQYWNKGEPNNIGEEDC	60
Human	AEEQNFLQLQSSRSNRFTWMGLSDLNQEGTWQWVDGSPLLPSFKQYWNRGEPNNVGEEDC	60
	*************************** ****************** ******* *****	
Cynomolgus macaque	AEFSGNGWNDDKCNLAKFWIC	81
Pig-tailed macaque	AEFSGNGWNDDKCNLAKFWIC	81
Rhesus macaque	AEFSGNGWNDDKCNLAKFWIC	81
Human	AEFSGNGWNDDKCNLAKFWIC	81
	*********************	

The solid box indicates residue Val351, which is crucial for AZN-D1 binding to DC-SIGN.

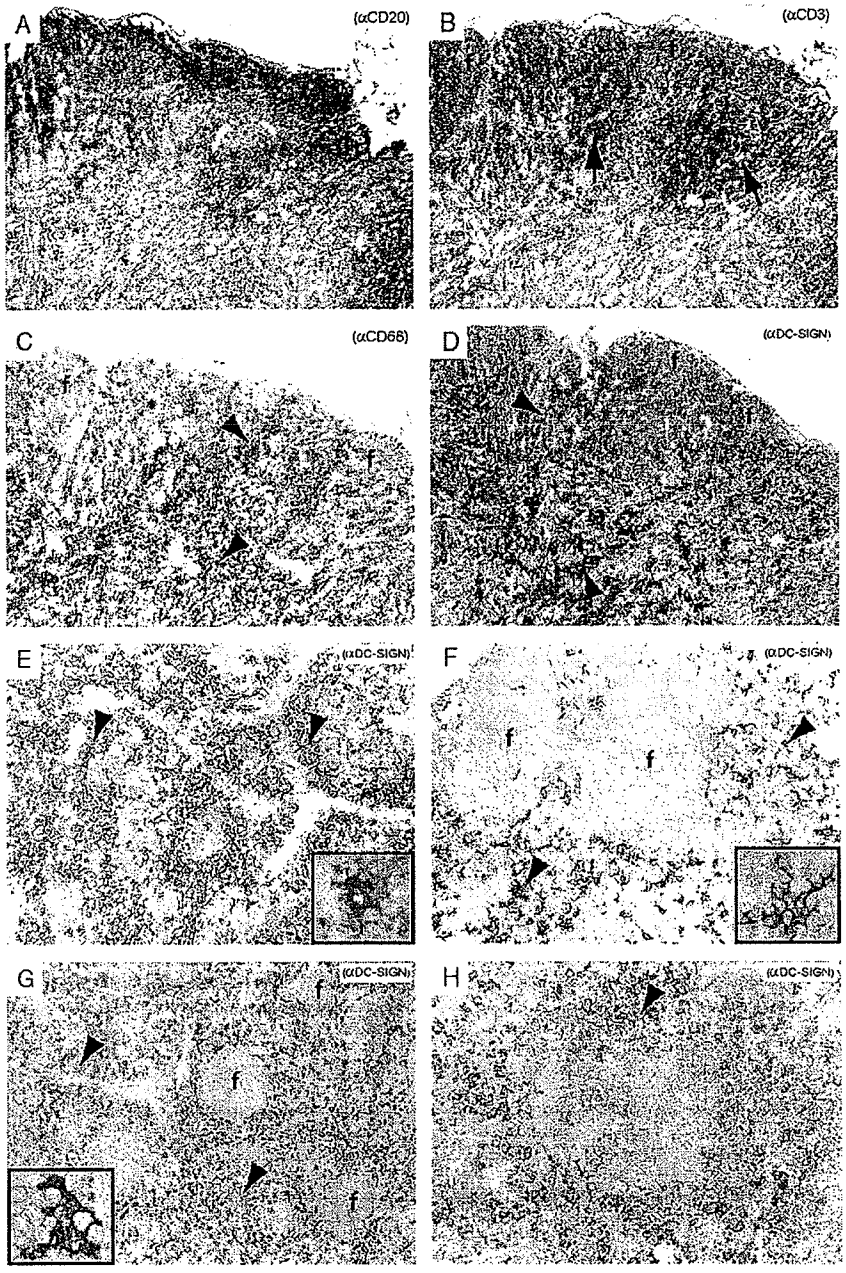

FIGURE 1. Expression patterns of (A) CD20 in B-cell follicles, (B) CD3 in paracortical areas, (C) CD68 on sinus cells, and (D–H) DC-SIGN in frozen serial sections of inguinal (A–D), auricular (E, F), or mesenteric (G–H) LNs from control cynomolgus macaques. Tissue sections were stained with the antibodies described above followed by biotinylated horse antimouse antibody. Magnification: 50 × (A–D); 100 × (G); 200 × (E, F, H); 630 × (insets). Arrowheads indicate sinuses, "f", follicles; arrows, paracortical areas.

The DC-SIGN–positive cells are large and irregular in shape, consistent with the morphology of myeloid cells (insets in Figs. 1E–G). Subsequent staining of consecutive sections with the myeloid marker CD68 (Fig. 1C) indicated that most DC-SIGN–positive cells in the sinuses and on the paracortical region are of myeloid origin. As expected, CD68 staining revealed a broader expression pattern than only DC-SIGN–positive cells. Next, sequential sections of the same auricular LNs were stained with antibodies directed against DC-SIGN (Figs. 2A, C), the mature DC marker DC-LAMP (Fig. 2B) or against MHC class II (Fig. 2D), which is present on both mature and immature DCs, and also B cells, interdigitating DCs and macrophages. The anti-DC-LAMP antibody stained only a few cells in the paracortical region and medullary sinuses (Fig. 2B) and the anti-MHC-II antibody labeled a large number of cells distributed throughout the LN (Fig. 2D). In summary, as previously described for rhesus macaques,[15] the expression pattern of DC-SIGN correlated well with that of CD68, but was less consistent with the expression patterns of the mature DC marker DC-LAMP or of MHC class II. These findings suggest that cynomolgus macaque DC-SIGN is

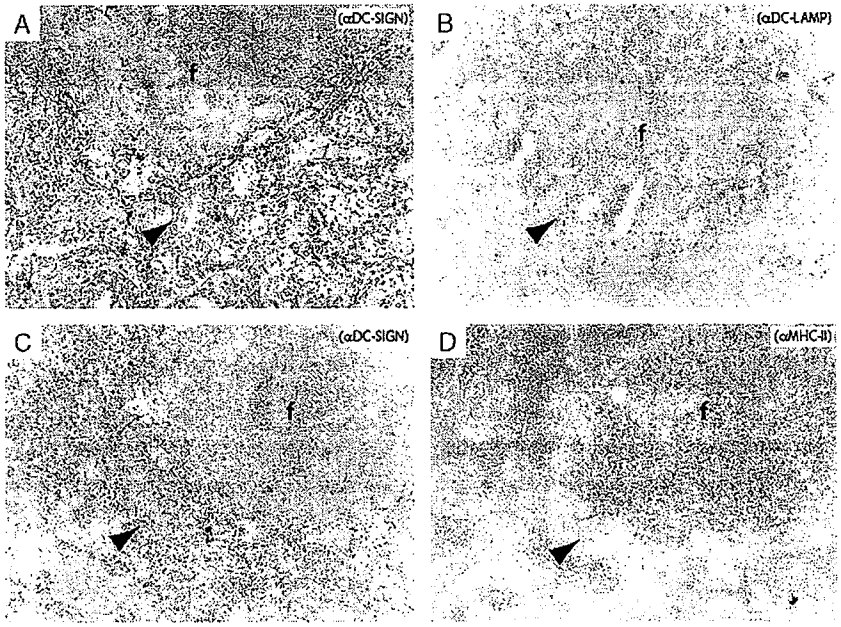

FIGURE 2. Expression patterns of (A, C) DC-SIGN on sinus cells, (B) DC-LAMP on scattered cells outside the follicles, (D) MHC class II in follicles in frozen serial sections of auricular LNs from control cynomolgus macaques. Tissue sections were stained with the antibodies described above followed by biotinylated horse antimouse antibody. Magnification: 100 ×. Arrowheads indicate sinuses; "f", follicles.

expressed on myeloid lineage cells such as macrophages and/or immature DCs.

Intravenous Administration of Anti-DC-SIGN Antibody Targets DC-SIGN–positive Cells in Lymphoid Tissues of Cynomolgus Macaques In Vivo

To determine whether and to what extent AZN-D1 could specifically target DC-SIGN–positive cells in vivo, cynomolgus macaques were treated with AZN-D1 delivered by intravenous injection. As it has previously been shown that mIgG1 delivered by intravenous injection (2 mg/kg/d for 12 consecutive days) into cynomolgus monkeys did not result in the detection of mIgG1-bound mononuclear cells within the LNs,[17] we did not euthanize more animals to include this control arm in our study. As subcutaneous injections only reach the draining LNs, AZN-D1 was delivered by intravenous injections to reach as many LNs as possible. Sections from several LNs of these macaques were stained with biotinylated horse antimouse antibody to assess the targeting efficiency of AZN-D1 in these tissues. Sections of auricular LNs showed massive numbers of AZN-D1–targeted cells (Figs. 3A–D). These AZN-D1–targeted cells were mainly present in the medullary sinuses, but were also evident in the afferent sinuses and in the paracortical area (Figs. 3A–D and Table 2). Sections of mesenteric LNs from cynomolgus macaques that were treated with AZN-D1 also showed numerous AZN-D1–targeted cells (Figs. 3E, F). Biotinylated horse antimouse antibody staining of sections from an untreated macaque did not demonstrate any background staining (data not shown).

To assess the percentage of DC-SIGN molecules that were targeted by AZN-D1, LN sections from cynomolgus macaques treated with AZN-D1 were stained with biotinylated AZN-D1. This biotinylated AZN-D1 antibody is as effective as unlabeled AZN-D1 in detecting DC-SIGN–positive cells in macaques (Figs. 4A, B), and allows visualization of the DC-SIGN molecules that remain unlabeled after the intravenous AZN-D1 administration. Strikingly, upon staining of sections of LNs from AZN-D1–treated macaques with biotinylated AZN-D1, no reactivity with the biotinylated antibody was observed (Fig. 4C). This indicates that essentially all DC-SIGN molecules present in these LNs have been targeted by the intravenously injected AZN-D1 antibody. Staining of LNs from AZN-D1–treated macaques with unlabeled AZN-D1 followed by biotinylated horse antimouse antibody (Fig. 4D) resulted in a similar staining pattern as observed with biotinylated horse antimouse antibody alone (Figs. 3A–D), confirming that all DC-SIGN molecules present in the LNs are targeted by AZN-D1.

Anti-DC-SIGN Antibody Targets Kupffer Cells in the Liver of Cynomolgus Macaques

Liver/LN-specific intracellular adhesion molecule-3-grabbing nonintegrin (L-SIGN or DC-SIGNR) is 77% identical to DC-SIGN, has a similar 3-dimensional structure and shares similar ligands. However, L-SIGN is expressed in specialized sinusoidal endothelial cells of the liver, LNs, and placenta, but not in DCs or macrophages. Sections of liver from cynomolgus macaques that were treated with AZN-D1 were stained with biotinylated horse antimouse antibody to assess the specificity of AZN-D1 in these tissues. Surprisingly, numerous AZN-D1–targeted cells were observed in the

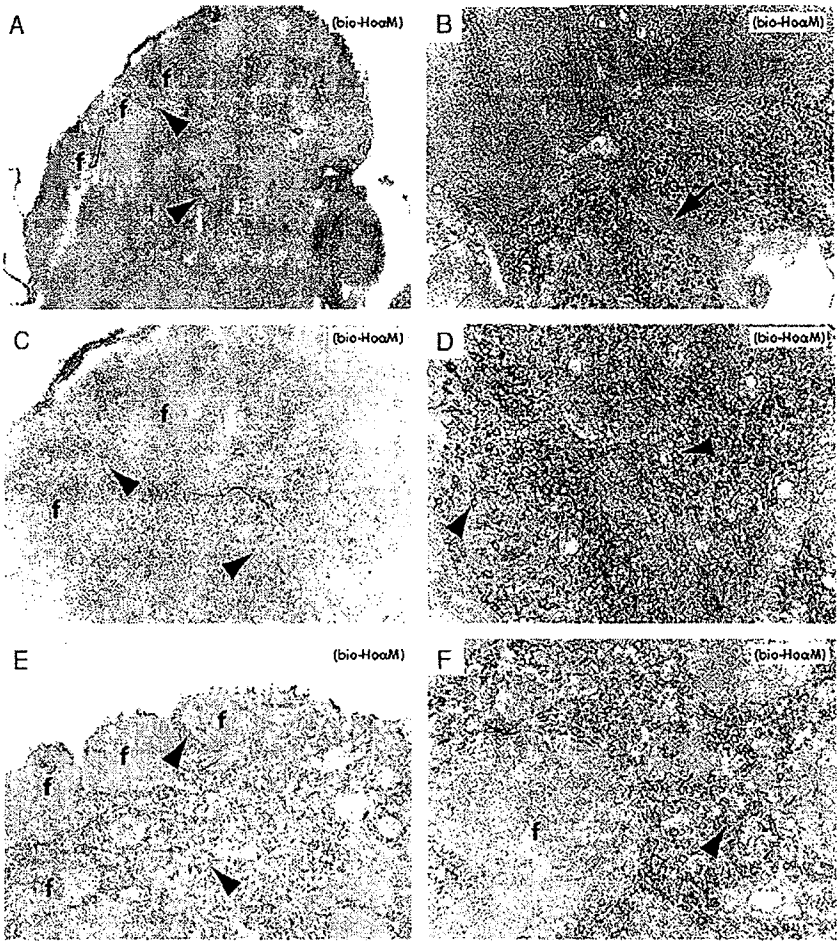

FIGURE 3. Cynomolgus macaques administered AZN-D1 in vivo have AZN-D1–targeted cells within (A–D) auricular and (E, F) mesenteric LNs. Frozen tissue sections were only stained with biotinylated horse antimouse antibody (bio-HoαM). Magnification: 50 × (A, E); 100 × (B, C); 200 × (D, F). Arrowheads indicate sinuses; "f", follicles; arrow, the hilum.

hepatic sinusoids in the liver of these animals (Fig. 5). To determine which cell types were targeted by AZN-D1, serial sections of livers from control cynomolgus macaques were stained with antibodies against human DC-SIGN (AZN-D1, Fig. 6A), DC-SIGN/L-SIGN (AZN-D3, Fig. 6B), CD68 (expressed on Kupffer cells or liver macrophages) (Fig. 6C), and CD31 (expressed on liver sinusoidal endothelial cells (LSECs) (Fig. 6D). The staining patterns of DC-SIGN and L-SIGN (obtained with AZN-D1 and AZN-D3) were similar to that observed after labeling with biotinylated horse antimouse antibody alone (Fig. 5). Interestingly, these staining

TABLE 2. Distribution of In Vivo-targeted AZN-D1 in the Draining LNs of Cynomolgus Macaques

Cynomolgus macaque	Treatment	Lymphoid Follicles	Paracortical Area	Medullary Sinuses	Afferent Sinuses
1	None	–	–	–	–
2	Oxazolone	–	–	–	–
3	Oxazolone	–	–	–	–
4	Oxazolone	–	–	–	–
5	Oxazolone	–	–	–	–
6	Oxazolone + AZN-D1	–	+	+ + +	+ +
7	Oxazolone + AZN-D1	–	+	+ + +	+ +
8	Oxazolone + AZN-D1	–	+	+ + +	+ +
9	Oxazolone + AZN-D1	–	+	+ + +	+ +

Animal 1 received no treatment. Animals 2 to 9 were treated intracutaneously with 2.5% oxazolone on days 3 to 5. Animals 6 to 9 also received intravenous injections of 3 mg/kg/d of AZN-D1 on days 1 to 7. The animals were euthanized on day 8. Frozen tissue sections from the draining (auricular) LNs of these macaques were stained with biotinylated horse antimouse antibody. Average staining intensity and extent of staining were scored: "–", negative; "+", < 5% positive cells; "+ +", 5% to 40% positive cells; "+ + +", > 40% positive cells.

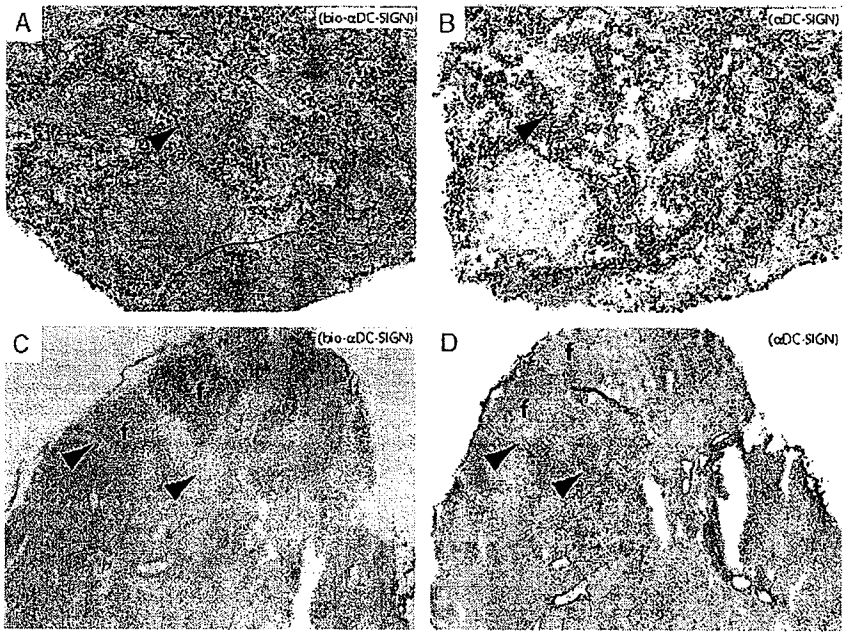

FIGURE 4. Unlabeled DC-SIGN molecules in the auricular LNs of cynomolgus macaques administered AZN-D1 in vivo. Serial frozen tissue sections from control cynomolgus macaques (A, B) or cynomolgus macaques treated with AZN-D1 (C, D) were stained with biotinylated AZN-D1 (bio-αDC-SIGN) alone (A, C) or unlabeled AZN-D1 (αDC-SIGN) followed by biotinylated horse antimouse antibody (B, D). Magnification: 50 × (C, D); 100 × (A, B). Arrowheads indicate sinuses; "f", follicles.

patterns were most consistent with that of anti-CD68 but not with the staining pattern of anti-CD31. These results are in contrast to those observed on human liver, in which AZN-D1 labels very few liver DCs (Fig. 7A) and AZN-D3 labels LSECs (Fig. 7B). As expected, staining of human liver with antibodies against CD68 (Fig. 7C) and CD31 (Fig. 7D) labeled Kupffer cells and LSECs, respectively. These results suggest that AZN-D1 is targeting Kupffer cells in the liver of cynomolgus macaques.

DISCUSSION

To our knowledge, this is the first study that demonstrates in vivo targeting of DC-SIGN–positive cells after the administration of an anti-DC-SIGN antibody. As expected, we have shown that cells targeted with the antihuman DC-SIGN monoclonal antibody AZN-D1 demonstrate the same distribution pattern in LNs as DC-SIGN–positive cells, which indicates that intravenous injection of AZN-D1 specifically targeted DC-SIGN–positive cells. These cells are mainly located in the LN medullary sinuses, although some cells can also be found in the afferent sinuses and on the paracortical or T-cell areas. As immunophenotyping of the DC-SIGN–positive cells in cynomolgus macaques is hampered by the absence of DC-specific or macrophage-specific antibodies, it remains difficult to unequivocally distinguish cynomolgus macaque DCs from macrophages. Although some studies have shown that DC-SIGN is mainly expressed by DCs,[1,3,18] recent studies have shown that DC-SIGN is expressed on medullary sinus macrophages in the paracortical area of normal human and rhesus macaque LNs.[2,15] Since several chimpanzee DC-SIGN alleles have been described,[19] it is tempting to speculate that human or cynomolgus macaque DC-SIGN alleles may also exist and that these alleles may account for the differences observed between different studies. Future studies are needed to clarify this hypothesis. Notwithstanding, both DCs and macrophages are APCs that can induce powerful and specific immune responses against foreign antigens and also induce cross-presentation of exogenous antigens on MHC class I molecules.[20 22] Irrespective of the precise

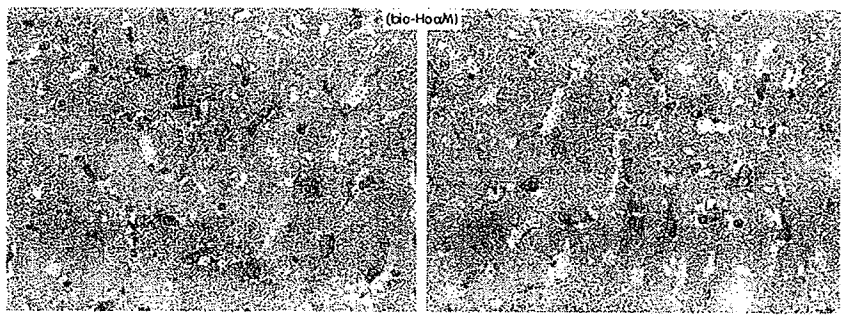

FIGURE 5. Cynomolgus macaques administered AZN-D1 in vivo have AZN-D1–targeted cells in the liver. Frozen tissue sections were stained with biotinylated horse antimouse antibody (bio-HoαM). Two representative sections are shown. Magnification: 400 × .

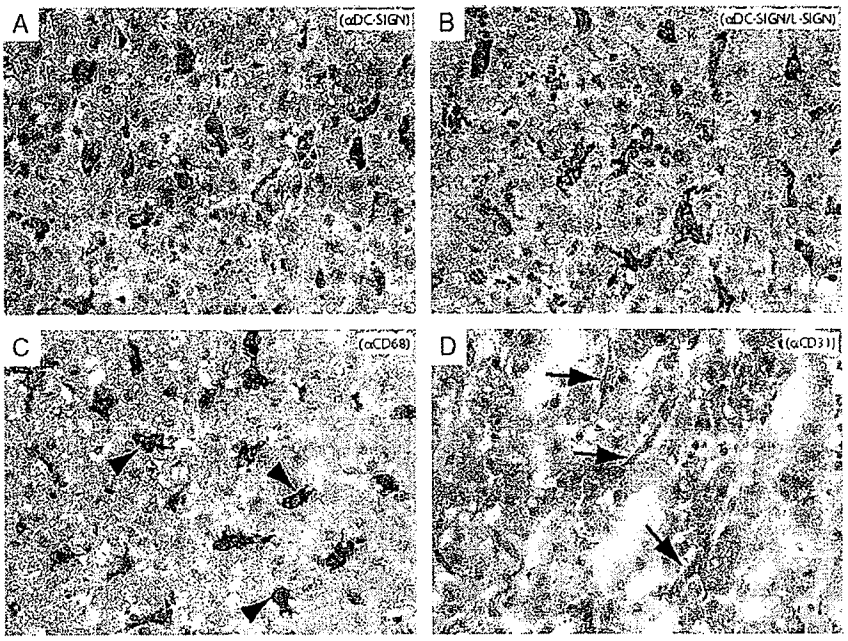

FIGURE 6. Frozen serial sections of liver tissue from control cynomolgus macaques were stained with (A) AZN-D1 (αDC-SIGN), (B) AZN-D3 (αDC-SIGN/L-SIGN), (C) anti-CD68 antibody for Kupffer cells, or with (D) anti-CD31 antibody for LSECs followed by biotinylated horse antimouse antibody. Magnification: 400 ×. Arrowheads indicate Kupffer cells; arrows, LSECs.

identity of these DC-SIGN–positive cells, our data demonstrate that DC-SIGN–positive APCs can be efficiently targeted in vivo with an antihuman DC-SIGN–specific mouse monoclonal antibody. Essentially all DC-SIGN molecules present in the LNs of AZN-D1–treated macaques were targeted with this antibody using the described intravenous treatment regimen. Others have shown that intravenous administration of mIgG1 (2 mg/kg/d for 12 consecutive days) into cynomolgus monkeys does not result in the detection of the mouse antibody on LN mononuclear cells.[17] Therefore, it seems unlikely that AZN-D1 treatment resulted in nonspecific uptake of the antibody or a down-regulation of DC-SIGN molecules on APCs in the LNs. Although DC-SIGN is a recycling receptor and the macaques were euthanized 24 hours after the last dose of AZN-D1, the administered dose of AZN-D1 (3 mg/kg/d) for 7 days resulted in a plasma concentration of AZN-D1 that was high enough to occupy all DC-SIGN molecules for 24 hours after the last dose. This dose of AZN-D1 was used

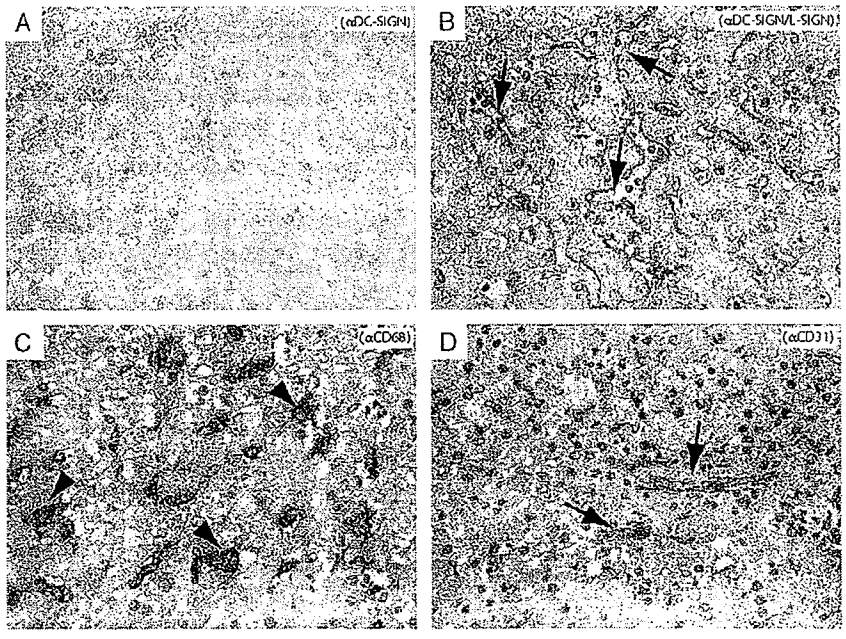

FIGURE 7. Frozen serial sections of liver tissue from a human biopsy were stained with (A) AZN-D1 (αDC-SIGN), (B) AZN-D3 (αDC-SIGN/L-SIGN), (C) anti-CD68 antibody for Kupffer cells, or with (D) anti-CD31 antibody for LSECs followed by biotinylated horse antimouse antibody. Magnification: 400 ×. Arrowheads indicate Kupffer cells; arrows, LSECs.

as a proof-of-principle to show that anti-DC-SIGN antibodies can target a significant number of DC-SIGN molecules in vivo and can reach the LNs. Subsequent in vivo studies can now be designed to determine the immunologic consequences of targeting DC-SIGN in cynomolgus macaques. We note that no apparent pathologic effects were observed in this study after a short-term administration of AZN-D1, but this should be further evaluated in additional studies in which antibody dose and frequency of administration are systematically examined.

As the DC-SIGN homolog L-SIGN is mainly expressed on LSECs in humans, we also assessed the specificity of AZN-D1 for APCs in the liver of cynomolgus macaques. Surprisingly, in contrast to results using human liver in which only a few scattered APCs stained with AZN-D1, immunohistochemical analysis of the reactivity of AZN-D1 on liver sections from control cynomolgus macaques revealed numerous labeled cells. As previously described for Rhesus macaques,[15] the staining pattern observed with AZN-D1 was similar to that of an anti-CD68 antibody, which labels the liver macrophages (Kupffer cells). Similarly, the anti-L-SIGN antibody AZN-D3 did not label LSECs, as was observed in the case of human liver, but rather, labeled cells with a very different morphology. Analysis of the AZN-D3 staining pattern was also very different from the staining pattern observed with an anti-CD31 antibody, which labeled LSECs in both human and control cynomolgus macaque liver. This fundamental difference in the liver staining pattern between human and both cynomolgus and rhesus monkeys may be either due to structural differences in the L-SIGN/DC-SIGN proteins themselves or to different expression patterns of DC-SIGN and L-SIGN in cynomolgus macaque liver compared with human liver. As AZN-D1 is capable of recognizing DC-SIGN in the LNs of these macaques, it is reasonable to assume that the antibody specificity is the same, but the expression patterns of DC-SIGN and L-SIGN in the liver of cynomolgus macaques are different from human liver.[16]

In agreement with these observations, cynomolgus macaques treated with AZN-D1 had AZN-D1–targeted cells in their livers. As was seen in control cynomolgus macaques, the staining pattern of these AZN-D1–targeted cells closely correlated with the distribution of Kupffer cells, as revealed by reactivity with an anti-CD68 antibody. It is unlikely that the AZN-D1 labeling in vivo is due simply to nonspecific binding of the antibody to Fc receptors on Kupffer cells, as liver sections from control macaques did not show Kupffer cell staining using an isotype-matched antibody control (data not shown), but did show reactivity with both anti-DC-SIGN and anti-L-SIGN antibodies. Instead, our results suggest that AZN-D1 is binding to a DC-SIGN homolog on Kupffer cells in cynomolgus monkeys.

In conclusion, this study provides proof-of-principle that an anti-DC-SIGN monoclonal antibody administered in vivo is capable of targeting APCs in LNs and in the liver. Recent in vitro studies have already shown that targeting antigens to APCs via a humanized anti-DC-SIGN antibody effectively induces antigen-specific immune responses,[5] and that MHC class II-restricted helper peptides and proteins targeted through DC-SIGN generate primary immune responses (Anke Kretz-Rommel, unpublished results). As has been described previously for targeting of DCs with anti-DEC-205 antibodies in mice,[9,10,12] future in vivo immunotherapeutic studies with anti-DC-SIGN antibodies, including humanized anti-DC-SIGN antibodies,[5] should explore the effect of APC maturation signals on induction of immunity or tolerance against the antigens targeted via DC-SIGN.

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